Determination of glucose level needed to induce aflatoxin production in *Aspergillus parasiticus*

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Previous studies have established that aflatoxin production is induced by high levels of glucose (or other compatible carbohydrates). However, the minimal amount of glucose needed to achieve this effect had not been determined. *Aspergillus parasiticus* was induced to produce aflatoxin when incubated for 18 h in the presence of ≥0.1 *M* glucose before addition of cycloheximide. Toxin formation was not induced at any glucose concentration in the mycelia incubated for 8 h. These results suggest that aflatoxin synthesis would not be expected in substrate containing <0.1 *M* of a carbohydrate source.

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Des études antérieures ont démontré que la production d'aflatoxine est induite par des taux élevés de glucose (ou d'autres hydrates de carbones compatibles). Cependant, la quantité minimale de glucose requise pour atteindre cet effet n'a pas été déterminée. En présence de ≥0,1 *M* de glucose, avant l'ajout de cycloheximide, *Aspergillus parasiticus* a été induit à produire de l'aflatoxine lorsqu'incubé pendant 18 h. La formation de la toxine n'a pas été induite chez les mycelia incubés pendant 8 h à aucune des concentrations de glucose utilisées. Ces résultats suggèrent qu'il n'y aurait pas de synthèse d'aflatoxine dans des substrats contenant une source d'hydrate de carbone de <0,1 *M*.

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Introduction

Aflatoxin synthesis by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* is highly dependent on a variety of nutritional factors. Sources of carbohydrates are particularly important since carbohydrates provide the two-carbon precursors for toxin synthesis. For this reason, a number of investigators have studied sugars and related carbohydrates that support fungal growth and aflatoxin production (Abdollahi and Buchanan 1981*a*, 1981*b*; Buchanan and Stahl 1984; Prasad 1983). Additional studies have indicated that the carbohydrate source is responsible for the induction of aflatoxin production (Abdollahi and Buchanan 1981*a*, 1981*b*; Buchanan and Stahl 1984; Buchanan et al. 1987). However, the minimal level of carbohydrate needed to achieve induction of aflatoxin production has never been assessed. Accordingly, the objective of this study was to determine the minimal concentration of glucose needed to induce aflatoxin production in *A. parasiticus* NRRL 2999.

One of the primary metholological considerations was the possibility that low concentrations of glucose could induce biosynthetic capabilities, but be insufficient to supply enough precursor for detectable levels of aflatoxin production. This potential problem area was circumvented by employing a modification of the replacement culture technique of Abdollahi and Buchanan (1981*a*) coupled with the use of a protein synthesis inhibitor. Peptone mineral salts broth (PMS) was prepared as described previously (Buchanan and Lewis 1984), adjusted to pH 4.5, transferred in 250-mL portions to 1000-mL flasks, and autoclaved for 20 min at 121°C. Each PMS flask was inoculated with sufficient volume of spore suspension (Buchanan and Lewis 1984) to achieve an inoculum of 4×10^3 conidia/mL and incubated at 28°C on a rotary shaker (120 rpm) for 48 h. The mycelia were then collected on cheesecloth, disrupted in a blender, and recollected as described previously (Buchanan and Lewis 1984). The homogenized mycelia were divided into 10 equal portions, transferred to 10 new 1000-ml flasks containing 250 mL of PMS, and reincubated at 28°C and 120 rpm for 24 h.

As expected from earlier investigations (Abdollahi and Buchanan 1981*a*; Buchanan and Lewis 1984; Buchanan and Stahl 1984), the mold achieved considerable growth as a result of the two periods of incubation in PMS, but did synthesize aflatoxins. These mycelia were collected on cheesecloth, rinsed 3 times with 0.85% KCl, and transferred in 250-mg portions to sets of 50-mL flasks containing 10 mL of PMS supplemented with 0.0, 0.001, 0.01, 0.1, or 0.33 *M* glucose. The 0.0 and 0.33 *M* supplementation levels served as negative and positive controls, respectively, in that 0.33 *M* glucose is known to induce toxin production (Abdollahi and Buchanan 1981*a*; Buchanan and Lewis, 1984; Buchanan and Stahl 1984; Buchanan et al. 1987). All flasks were incubated for 18 h at 28°C and 120 rpm.

The mycelia were then transferred to individual 50-mL flasks containing 10 mL of PMS supplemented with 0.33 *M* glucose and 200 μg cycloheximide/mL (PMS–GC). This level of glucose is ample for the production of aflatoxins, while the cycloheximide prevents any further *de novo* synthesis of enzymes associated with the aflatoxin biosynthetic pathway (Abdollahi and Buchanan 1981*a*; Buchanan et al. 1987). One set of four replicate cultures for each glucose concentration was analyzed immediately after transfer for medium pH, aflatoxins, and mycelial dry weights. A second set of cultures was incubated for 48 h at 28°C and 120 rpm, and then analyzed. The entire experiment was run on two separate occasions. An additional experiment was performed where the duration of the incubation period used in conjunction with the PMS supplemented with various levels of glucose was 8 instead of 18 h. That experiment used five replicate cultures per glucose concentration, but was not duplicated on a separate occasion.

The cultures (mycelium + medium) were analyzed for aflatoxins (B_1, B_2, G_1, G_2) using thin-layer chromatographic techniques as described previously (Buchanan et al. 1986). After extraction, mycelia were transferred to tared aluminum dishes, dried at 80°C for 24 h, and mycelial dry weights determined gravimetrically.

TABLE 1. Aflatoxin production by mycelia of *A. parasiticus* preincubated for 18 h in PMS medium supplemented with varying concentrations of glucose and then transferred to PMS–GC with 200 μg/mL cycloheximide[a]

Glucose content of preincubation medium (*M*)	Post-transfer incubation							
	0 h				48 h			
	pH	Mycelium dry weight (mg)	Aflatoxin/culture (μg)	Aflatoxin/mycelium (ng/mg)	pH	Mycelium dry weight (mg)	Aflatoxin/culture (μg)	Aflatoxin/mycelium (ng/mg)
0.000	4.65	48.2(0.03)	ND	—	5.70	43.3(2.2)	ND	—
0.001	4.62	53.4(2.1)	ND	—	5.67	44.8(1.8)	ND	—
0.010	4.59	52.3(2.1)	ND	—	5.25	46.4(2.3)	ND	—
0.100	4.55	55.6(4.8)	0.07(0.04)	1.45(0.98)	4.61	62.2(3.5)	2.06(0.53)	33.1(9.09)
0.330	4.55	59.2(3.4)	0.16(0.16)	2.40(2.40)	4.53	69.0(0.2)	2.40(0.2)	33.6(18.9)
0.330 (control)[b]	4.55	59.2(3.4)	0.16(0.16)	2.40(2.40)	3.98	412.4(46.7)	94.70(28.30)	229.6(84.8)

NOTE: Values represent $\bar{x}$(± SEM), n = 8 replicate cultures. ND, none detected; lower limit of detection <10 ng/culture.
[a]Initial pH = 4.50.
[b]Transferred to PMS–GC without cycloheximide.

TABLE 2. Aflatoxin production by mycelia of *A. parasiticus* preincubated for 8 h in PMS medium supplemented with varying concentrations of glucose and then transferred to PMS–GC with 200 μg cycloheximide/mL

Glucose content of preincubation medium (*M*)	Post-transfer incubation					
	0 h			48 h		
	pH[a]	Mycelium dry weight (mg)	Aflatoxin/culture (μg)	pH	Mycelium dry weight (mg)	Aflatoxin/culture (μg)
0.000	4.59	68.2(3.4)	ND	3.90	46.6(2.0)	ND
0.001	4.58	74.0(4.4)	ND	3.92	50.9(2.8)	ND
0.010	4.54	65.5(3.3)	ND	4.22	53.8(1.4)	ND
0.100	4.56	77.6(3.1)	ND	4.21	51.4(1.9)	ND
0.330	4.61	67.4(5.6)	ND	4.25	44.1(1.9)	ND

NOTE: Values represent $\bar{x}$(± SEM), n = 5 replicate cultures.
[a]Initial pH = 4.50.

Aflatoxins were not detected in cultures preincubated in PMS supplemented with ≤0.01 *M* glucose (Table 1). Low levels of aflatoxins were detected immediately after transfer to PMS–GC for those cultures initially incubated in ≥0.1 *M*. Greater amounts of aflatoxins were detected after 48 h of incubation in PMS-GC, indicating *de novo* aflatoxin production. These cultures also had increased dry weights as compared with cultures preincubated in glucose concentration ≤0.01 *M*. Differences in pH among these groups of cultures were also noted. Control cultures preincubated in PMS with 0.33 *M* glucose and then incubated in PMS–GC without cycloheximides had increased mycelial dry weights and aflatoxin production as compared with the corresponding PMS–GC cultures.

When mycelia were preincubated in PMS with varying levels of glucose for 8 instead of 18 h, aflatoxin production was not detected in any of the PMS–GC cultures (Table 2). Differences in mycelial dry weights of the 48-h cultures were not detected among various preincubation glucose levels. However, an overall decrease was observed in comparison with the cultures analyzed immediately after transfer. This decline in mycelial dry weights was similar to that observed previously with cultures preincubated for 18 h in media containing ≤0.01 *M* glucose. The pH of the 8-h preincubation cultures was slightly depressed after 48 h incubation in PMS–GC for those mycelia initially preincubated with glucose levels of ≤0.001 *M* glucose.

The transfer protocol employed in this study allowed separation of the actions of glucose as both an inducer and substrate for aflatoxin synthesis. The results provide additional information about the carbohydrate-mediated induction of aflatoxin synthesis, indicating that a combination of relatively high levels (≥0.1 *M*) of glucose together with an extended induction period are required to induce aflatoxin production. The time requirements for induction of aflatoxin production are in general agreement with previous studies (Abdollahi and Buchanan 1981*a*; Buchanan et al. 1987) that found that cycloheximide blocks *de novo* aflatoxin production if added to cultures within 6–10 h of the mold having been transferred to a glucose-containing medium.

Production of aflatoxin by cultures preincubated for 18 h in medium containing ≥0.1 *M* glucose appears to correlate with small increases in mycelial dry weight during the 48-h incubation in PMS–GC. Similar increases were not observed for cultures preincubated with glucose levels ≤0.01 *M* nor were increases observed in conjunction with any glucose concentration for mycelia preincubated for 8 h. Buchanan and Lewis (1984) suggested that increases in the dry weights observed when pregrown mycelia are incubated under nongrowing

conditions may largely reflect the accumulation of secondary metabolites by *A. parasiticus*.

Differences in the pH values of PMS–GC after 48 h of post-transfer incubation appear to correlate with the induction of aflatoxin synthesis during the 18-h preincubation. The pH rise in cultures containing mycelia preincubated in medium containing ≤ 0.01 *M* glucose suggests that these fungal cultures were actively catabolizing amino acids. Alternatively, cultures induced to produce aflatoxins maintained the initial pH values. This indicates amino acid catabolism was not occurring or was counterbalanced by formation of organic acids. However, cultures employing mycelia preincubated for 8 h in the presence of different glucose concentrations showed a slight decline in pH. This suggests that the glucose present was being catabolized to organic acids. No direct relationship between changes in pH of the cultures and the induction of aflatoxin synthesis was apparent by comparison of the 8- and 18-h preincubation cultures. The reasons for the differences between the 8- and 18-h cultures needs clarification and will require additional study.

In summary, these results demonstrate that the glucose-mediated induction of aflatoxin synthesis is dependent on the concentration of glucose. The fungal cells need to be exposed to relatively high concentrations of the carbohydrate. The induction process required relatively long exposure times which may indicate a need for sufficient accumulation and (or) metabolism of carbohydrate sources. The results suggest further that little, if any, aflatoxin production would be expected in substrates containing less than 0.1 *M* of a suitable carbohydrate source. Confirmation of this supposition will require additional studies with natural substrates having varying levels of carbohydrates.

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